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A novel function of peroxiredoxin 1 (Prx-1) in apoptosis signal-regulating kinase 1 (ASK1)-mediated signaling pathway

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Abstract **We report a novel function of peroxiredoxin-1 (Prx-1) in the ASK1-mediated signaling pathway. Prx-1 interacts with ASK1 via the thioredoxin-binding domain of ASK1 and this interaction is highly inducible by H_2O_2. However, catalytic mutants of Prx1, C52A, C173A, and C52A/C173A, could not undergo H_2O_2 inducible interactions, indicating that the redox-sensitive catalytic activity of Prx-1 is required for the interaction with ASK1. Prx-1 overexpression inhibited the activation of ASK1, and resulted in the inhibition of downstream signaling cascades such as the MKK3/6 and p38 pathway. In Prx-1 knockdown cells, ASK1, p38, and JNK were quickly activated, leading to apoptosis in response to H_2O_2. These findings suggest a negative role of Prx-1 in ASK1-induced apoptosis.**

Keywords: Apoptosis; Apoptosis signal-regulating kinase 1; Peroxiredoxin 1; Hydrogen peroxide; Mitogen-activated protein kinase

1. Introduction

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family that activates both MKK4/MKK7-c-Jun NH_2-terminal kinase (JNK) and MKK3/MKK6-p38 MAPK signaling cascades [1–3]. ASK1 activation regulates various cellular events such as differentiation and apoptosis. Overexpression of the wild-type or the constitutively active form of ASK1 has been reported to induce apoptosis in various cell types [3–5]. On the other hand, moderate expression of a constitutively active form of ASK1 induces neuronal differentiation or even survival in PC12 cells [6]. Moreover, low and high expression of exogenous ASK1 in keratinocytes induces differentiation and apoptosis, respectively [7]. Although the precise mechanism by which ASK1 regulates these distinctly different processes has not yet been elucidated, multiple factors, such as cell line specificity, cellular reduction/oxidation (redox) regulatory protein and other unknown factors, seem to be involved.

Under non-stressed conditions, ASK1 is inhibited by association with its physiological inhibitor, thioredoxin (Trx) [8,9]. Trx directly binds to the N-terminal non-catalytic region of ASK1. When cells are exposed to hydrogen peroxide (H_2O_2) or tumor necrosis factor alpha (TNF-α), H_2O_2-dependent oxidation of Trx occurs, which causes dissociation of Trx from ASK1 and subsequent activation of ASK1 [9]. Another cellular function of Trx is to reduce oxidized 2-Cys peroxiredoxin (Prxs) proteins, which were recently defined as redox regulatory proteins capable of eliminating intracellular H_2O_2 [10,11]. In the catalytic intracellular H_2O_2 removal cycle of 2-Cys Prx proteins, the conserved, H_2O_2-sensitive cysteine residue (Cys^{52} in mammalian peroxiredoxin 1 (Prx-1)) is selectively oxidized by H_2O_2 to Cys-SOH, which then reacts with the COOH-terminal conserved Cys-SH (Cys^{173} in Prx-1) of the other subunit in the homodimer to form an intermolecular disulfide [10,12]. The disulfide is subsequently and specifically reduced by Trx. However, it has not been investigated whether Prxs are involved in ASK1-mediated signaling as redox regulatory proteins. Here we report for the first time that Prx-1 negatively regulates ASK1 activity in a redox-sensitive manner.

2. Materials and methods

2.1. Cell culture and cytokines

Human embryonic kidney 293 cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing a high concentration of glucose (4.5 mg/ml), supplemented with 10% fetal bovine serum and 100 units/ml penicillin, in a 5% CO_2 atmosphere at 37 °C.

2.2. Antibodies

Monoclonal antibodies to HA, Flag, and Prx-1 were purchased from Roche, Stratagene, and Abcam, respectively. Anti-Myc, anti-Trx, anti-ASK1, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-Thr 845 ASK1, anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK, and anti-GAPDH antibodies were purchased from Cell Signaling Technology.

2.3. Plasmids

pcDNA3-HA-ASK1WT was kindly provided by Dr. Hidenori Ichijo. Myc-Prx-1 was generated by PCR, using a HeLa cDNA library as a template, and inserted into pcDNA3 with a Myc-tag. Myc-Prx-1 (C52A), Myc-Prx-1 (C173/A), and Myc-Prx-1 (C52A/C173/A) mutants were generated by site-directed mutagenesis. cDNAs encoding ASK1ΔC, ASK1-NT655, ASK1-NT384, and ASK1-NT277 were generated by PCR, using pcDNA3-HA-ASK1wt as a template, and inserted into pcDNA3 with a Flag-tag. Human Trx was generated by PCR, using a HeLa cDNA library as a template, and inserted into pcDNA3.

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Abbreviations: ASK1, apoptosis signal-regulating kinase 1; Prx-1, peroxiredoxin 1; Trx, thioredoxin; H_2O_2, hydrogen peroxide; shRNA, short hairpin RNA; JNK, c-Jun NH_2-terminal kinase; TNF-α, tumor necrosis factor alpha

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2.4. Transfection and apoptotic cell death

DNA transfections were performed using FuGENE6 (Roche Molecular Biochemicals) or MP-100 microPorator (Digital Bio, Korea) according to the respective manufacturer's instructions. Apoptotic cell death was measured by flow cytometry (FACS® Calibur; Becton Dickinson) with annexin V staining or by propidium iodide (PI) staining.

2.5. Immunoblotting analysis

Cells were lysed in lysis buffer (lysis buffer A) containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1.5% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Immunoblotting analysis was performed as previously reported [8,9].

2.6. Co-immunoprecipitation assay

To examine protein interaction in HEK293 cells, transfected cells were lysed. Thereafter, co-immunoprecipitation procedures were followed as previously reported [8,9].

2.7. Generation of Prx-1 shRNAs

Short hairpin RNAs (shRNAs) with human Prx-1 target sequence shRNA-1 (5′-ACAAGGAGGACTGGGACCC-3′), shRNA-2 (5′-GGAGGACTGGGACCCATGA-3′), and shRNA-3 (5′-ATGACCTCCCTGTTGGCCG-3′) were produced by chemically synthesized DNA oligonucleotides and cloned into pSUPER.retro vector according to the manufacturer's instruction (OligoEngine).

3. Results and discussion

3.1. H_2O_2 treatment strongly induces the interaction between ASK1 and Prx-1

To investigate the interaction of Prx-1 with ASK1 in a cellular context, HA-tagged ASK1 was transiently transfected in 293 cells together with expression plasmid encoding Myc-tagged Prx-1. After co-transfection for 48 h, the cells were stimulated with various concentrations of H_2O_2, extracted, and immunoprecipitated with anti-Myc antibody. HA-ASK1 was weakly co-immunoprecipitated with Myc-Prx-1 under the resting condition (Fig. 1A, lane 5). Following stimulations with H_2O_2, interestingly, the ASK1-Prx-1 interaction was gradually enhanced and picked at 5 mM H_2O_2 (Fig. 1A, lanes 6-8). To confirm whether this inducible interaction is occurred in endogenous level, immunoprecipitation assay was performed with anti-Prx-1 antibody in 293 cells treated or non-treated with 5 mM H_2O_2. Co-immunoprecipitation results indicated that the interaction between ASK1 and Prx-1 was appeared at the resting condition (Fig. 1B, lane 3), and significantly enhanced by stimulation with 5 mM H_2O_2 (Fig. 1B, lane 4). Similar results could be detected in HeLa cells (data not shown). These results indicate that the molecular association between ASK1 and Prx-1 is highly inducible in response to H_2O_2.

3.2. Prx-1 interacts with ASK1 via the N-terminal Trx-binding domain

We further identified Prx-1-interacting regions of ASK1. As shown in Fig. 2A, several binding domains in ASK1 have previously been identified [9]. Based on these results, we constructed four truncated mutants of Flag-tagged ASK1: ASK1ΔC, ASK1-NT655, ASK1-NT384 and ASK1-NT277. Flag-tagged mutant constructs of ASK1 were transiently transfected in 293 cells together with Myc-tagged Prx-1. After co-transfection for 48 h, cells were extracted and immunoprecipitated with anti-Myc antibody. Among the ASK1 constructs, only ASK1ΔC, but not ASK1-NT655, ASK1-NT384 or ASK1-NT277, was associated with Prx-1 in the resting condition (Fig. 2B, lanes 5). Since the ASK1-Prx-1 interaction was induced by H_2O_2 treatment (Fig. 1), we further addressed whether these constructs showed inducible interactions with Myc-Prx-1. When co-immunoprecipitation assays were performed after stimulation with 5 mM H_2O_2, five different constructs were significantly associated with Myc-Prx-1 (Fig. 2C, lanes 6–10), indicating that Trx-binding region of ASK1, aa 46–277, is necessary for inducible association with Prx-1.

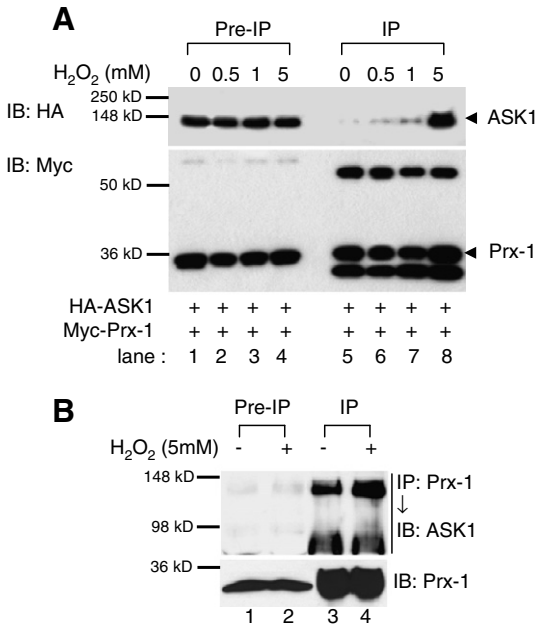

Fig. 1. Inducible interaction between Prx-1 and ASK1. (A) HEK293 cells were transfected for 48 h with the indicated combinations of plasmids encoding HA-ASK1 and Myc-Prx-1. Cells were stimulated with various concentrations of H_2O_2 for 20 min. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and the resulting Myc immunoprecipitates were subjected to immunoblot analysis with anti-HA antibody. (B) HEK293 (1.5×10^8) were treated with or without 5 m M H_2O_2 for 20 min. Cell lysates were subjected to immunoprecipitation with anti-Prx-1 antibody, and the resulting Prx-1 immunoprecipitates were subjected to immunoblot analysis with anti-ASK1 antibody.

3.3. Catalytic functions of Cys^{52} and Cys^{173} in Prx-1 are required for inducible interaction between Prx-1 and ASK1

The catalytic functions of Prx-1 to eliminate H_2O_2 are critically dependent on residues Cys^{52} in the N-terminal portion and Cys^{173} in the C-terminal portion [10,12] of the protein. If H_2O_2 is a critical inducer for mediating the interaction between ASK1 and Prx-1, the catalytic function of the Cys^{52} and Cys^{173} residues might be required for this interaction. Therefore, we examined this possibility. We constructed three mutant forms, C52A, C173/A, and C52A/C173A (Fig. 3A). HA-ASK1 was transiently transfected in 293 cells together with wild-type Myc-tagged Prx-1, Myc-tagged C52A Prx-1, Myc-tagged C173A Prx-1, or Myc-tagged C52A/C173A Prx-1. After co-transfection for 48 h, the cells were stimulated with 5 mM H_2O_2 for 20 min. Cells were extracted and immunoprecipitated with anti-Myc antibody. Consistent with Figs. 1 and

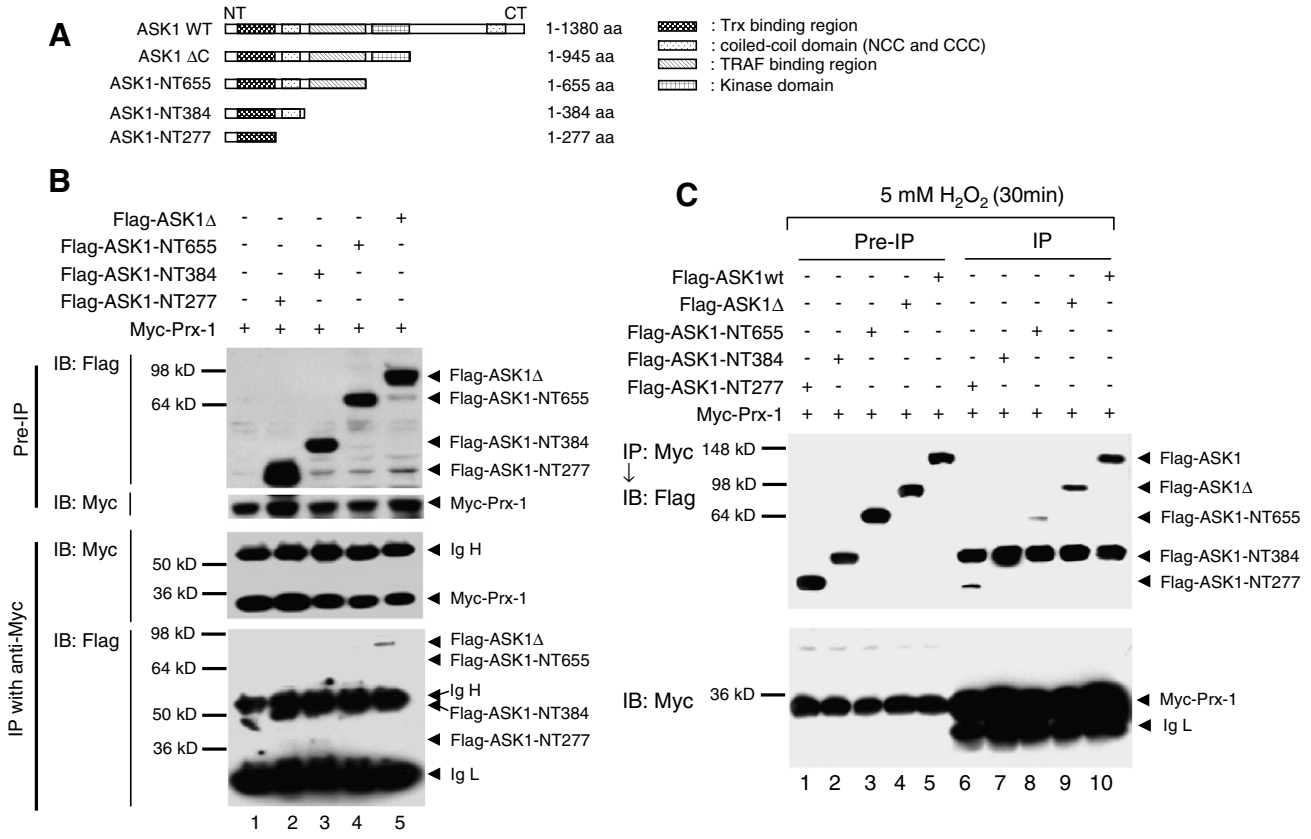

Fig. 2. Identification of Prx-1 interaction region of ASK1. (A) Schematic representation of ASK1WT and its C-terminal deletion mutants, with the amino acid numbers indicated. (B) HEK293 cells were transfected for 48 h with the indicated combinations of plasmids encoding Myc-Prx-1, Flag-ASK1Δ, Flag-ASK1-NT655, Flag-ASK1-NT-384, and Flag-ASK1-NT277. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and the resulting Myc immunoprecipitates were subjected to immunoblot analysis with anti-Flag antibody. (C) HEK293 cells were transfected for 48 h with the indicated combinations of plasmids encoding Myc-Prx-1, Flag-ASK1WT, Flag-ASKIΔ, Flag-ASK1-NT655, Flag-ASK1-NT384, and Flag-ASK1-NT277. Cells were treated with 5 mM H_2O_2 for 20 min. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and the resulting Myc immunoprecipitates were subjected to immunoblot analysis with anti-Flag antibody.

2, wild-type ASK1 was strongly co-immunoprecipitated with wild-type Prx-1 following stimulation with H_2O_2 (Fig. 3B, lanes 5). However, no significantly inducible interaction with ASK1 was detected with the C52A, the C173/A, or C52A/C173A construct (Fig. 3A, lanes 6–8). These results strongly suggest that catalytic functions of Prx-1 in response to H_2O_2 are essential for interaction with ASK1.

We further tested whether Prx-1-ASK1 interaction affects ASK1 activation. HA-ASK1 was transiently transfected in HeLa cells together with Myc-Prx-1. After co-transfection for 48 h, the cells were stimulated with 5 mM H_2O_2. Cells were extracted and immunoblotted with anti-phospho-Thr845 antibody. Under the resting condition, the phosphorylation of Thr845 was slightly detected (Fig. 3B, lane 2). Following stimulation with H_2O_2, however, the phosphorylation greatly increased (Fig. 3B, lane 6). Interestingly, the phosphorylation was markedly decreased by Prx-1 overexpression (up to two-folds), compared to that of without Prx-1 (Fig. 3B, lane 8). Furthermore, the inhibitory effect was critically affected on the signaling events downstream of ASK1. Phosphorylations of MKK3/6 and p38 were significantly inhibited by Prx-1 overexpression (Fig. 3B, lane 8), indicating that Prx-1 negatively regulates the ASK1-mediated signaling pathway for the activation of p38 pathway in response to H_2O_2.

3.4. Prx-1 functions as an endogenous antagonist against ASK1-mediated apoptosis by H_2O_2

It has been reported that ASK1 is required for H_2O_2-induced cell death [8]. Based on the data indicating that Prx-1 negatively regulates ASK1 activity, we hypothesized that endogenous Prx-1 may affect ASK1-induced apoptosis. To explore this question, we designed there shRNAs targeting endogenous Prx-1. Suppression of endogenous Prx-1 expression was tested by trasfecting the shRNAs in HeLa cells. ShRNA2, but not shRNA1 and shRNA3, efficiently suppressed the endogenous expression of Prx-1 (Fig. 4, lane 4). Similar results could be observed in 293 cells (data not shown). With shRNA-2, we further tested whether Prx-1-knockdown can affect the activation of ASK1-mediated signaling by H_2O_2. HeLa cells were transiently transfected with shRNA-2, and stimulated with 5 mM H_2O_2 for different times as indicated in Fig. 4B, and then phosphorylations of Thr845 of ASK1 and p38 were evaluated. Consistent with Fig. 4A, shRNA2 effectively suppressed endogenous expression of Prx-1 (Fig. 4B, lanes 5–8). Upon the stimulation with H_2O_2, the levels of phosphorylated p38 and JNK were gradually increased in a time dependent manner (Fig. 4B, lanes 1–4). Interestingly, the levels were quickly and strongly appeared at 15 min, and continuously maintained in Prx-1-knockdown

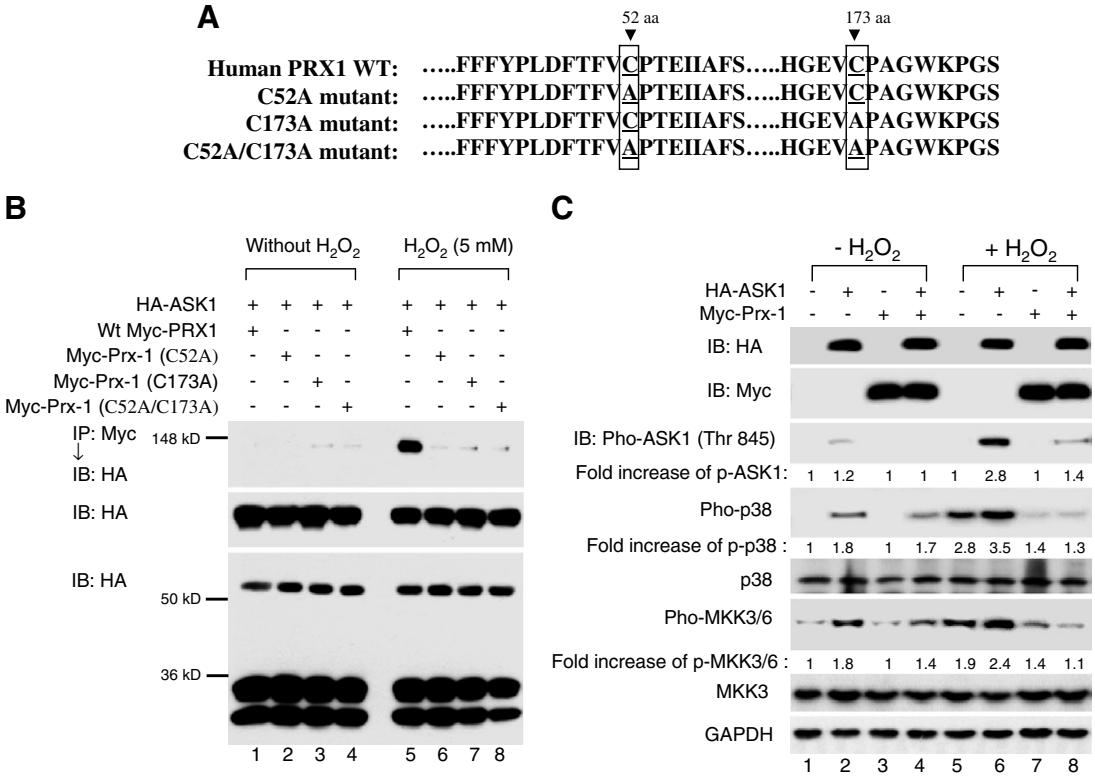

Fig. 3. The Prx-1-ASK1 is dependent on the catalytic functions of Cys^{52} and Cys^{173} of Prx-1 and inhibits ASK1-mediated signaling. (A) Based on the GenBank (NM 181696), we generated three mutant forms of Prx-1, C52A, C173A, and C52A/C173A mutants, by site-directed mutagenesis method. (B) HEK293 cells were transfected for 48 h with the indicated combinations of plasmids encoding HA-ASK1, Myc-Prx-1, Myc-Prx-1 (C51A), Myc-Prx-1 (C173A), and Myc-Prx-1 (C51A/C171A). Cells were treated with 5 mM H_2O_2 for 20 min. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and the resulting Myc immunoprecipitates were subjected to immunoblot analysis with anti-HA antibody. (C) HeLa cells were transfected for 48 h with the indicated combinations of plasmids encoding HA-ASK1 and Myc-Prx-1 and then treated with 5 mM H_2O_2 for 20 min. Cells lysates were subjected to immunoblot analysis with anti-phospho-Thr845 ASK1, anti-HA, anti-Myc, anti-phospho-p38, anti-p38, anti-MKK3/8, anti-MKK3, and anti-GAPDH antibodies. To quantitate the intensity of the bands, we use Image J provided from NIH.

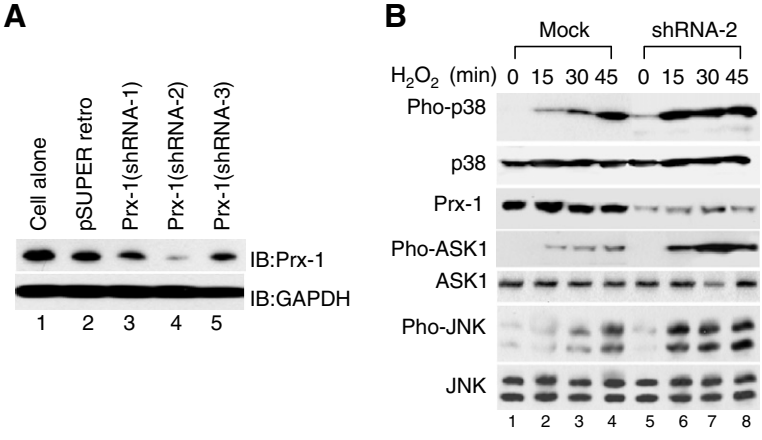

Fig. 4. Prx-1 negatively regulates ASK1-mediated signaling pathway for the activation of p38. (A) Using MP-100 microPorator, HeLa cells (5×10^5) were transfected with pSUPER.retro, shRNA-1, shRNA-2, and shRNA-3 vector. After transfection for 48 h, cell lysates were subjected to immunoblot analysis with anti-Prx-1 or anti-GAPDH antibody. (B) Using MP-100 microPorator, HeLa cells were tranfected with pSUPER retro (Mock) and shRNA-2. After transfection for 48 h, cultured cells were treated without or with 0.2 mM H_2O_2 for different times as indicated, and cell lysates were subjected to immunoblot analysis with anti-phospho-p38, anti-p38, anti-Prx-1, anti-phospho-Thr845 ASK1, anti-ASK1, anti-phospho-JNK, and anti-JNK antibodies.

cells (Fig. 4B, lanes 6–8). Similar patterns of the phophorylation of Thr845 of ASK1 could be detected in Prx-1-knock-down cells, compared with that of mock-transfected cells (Fig. 4B, lanes 2–4). These results strongly suggest that endog-

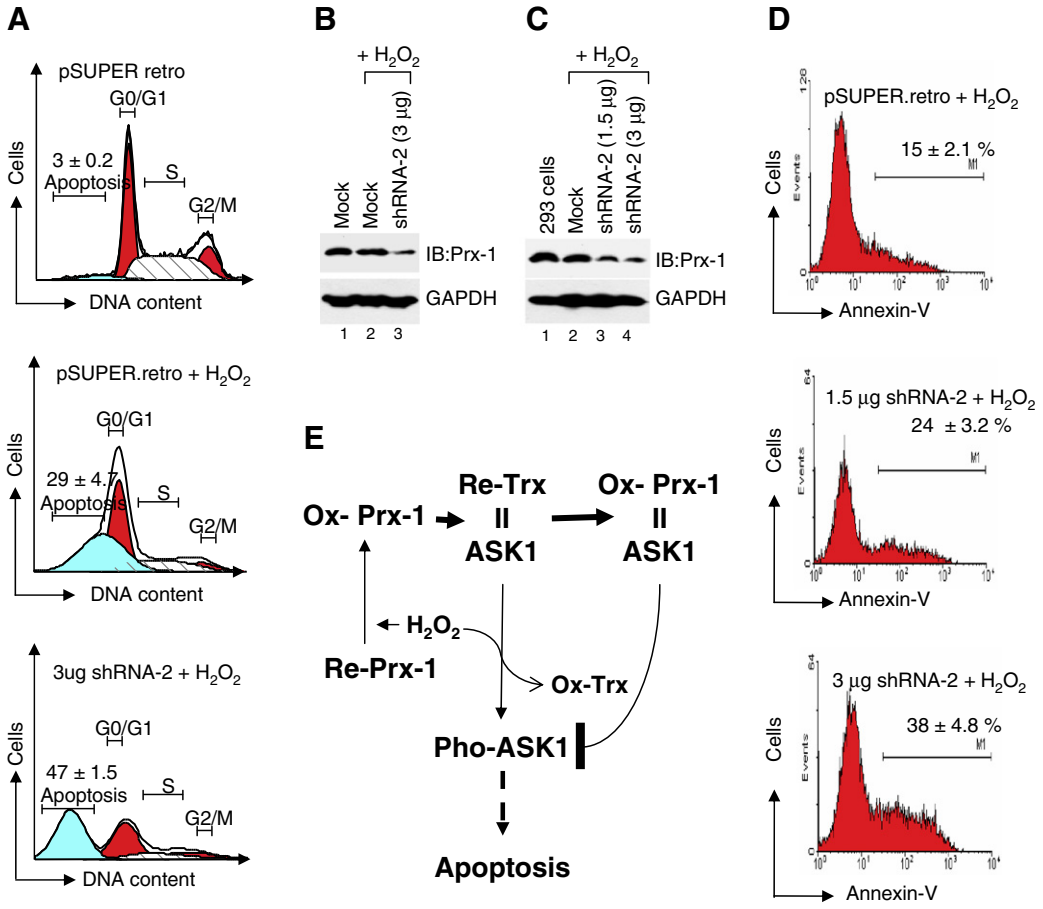

Fig. 5. H_2O_2-induced apoptosis is highly sensitive in Prx-1 -knockdown cells. (A) and (B) Using MP-100 microPorator, HeLa cells (5×10^5) were transfected with pSUPER.retro vector and shRNA-2 vector. After transfection for 48 h, cultured cells were treated without or with 0.2 mM H_2O_2 for 24 h. shRNA2-induced knockdown of endogenous Prx-1 was tested by immunoblot analysis with anti-Prx-1 (B). And, apoptotic cell death was measured by flow cytometry (A). Data are represented as the mean of triplicate independents ± S.E.M. (C) and (D) Using MP-100 microPorator, 293 cells (5×10^5) were transfected with pSUPER.retro vector or shRNA-2 vector with different concentrations. After transfection for 48 h, cultured cells were treated with 0.2 mM H_2O_2 for 24 h. shRNA2-induced knockdown of endogenous Prx-1 was tested by immunoblot analysis with anti-Prx-1 (C). Apoptotic cell death was measured by flow cytometry (D). Data are represented as the mean of triplicate independents ± S.E.M. (F) A suggested simplified model for the inhibition of Prx-1 in ASK1-mediated activation in response to H_2O_2 (Ox-Prx-1, oxidized Prx-1; Re-Prx-1, reduced Prx-1; Ox-Trx, oxidized thioredoxin; Re-Trx, reduced thioredoxin).

enous Prx-1 as a negative regulator play a key role for the ASK1-meidated signaling to activate p38 and JNK pathway in response to H_2O_2.

We next examined functional roles of Prx-1 in H_2O_2-induced apoptosis. HeLa cells were transiently transfected with shRNA2. After transfection for 48 h, the cells were treated with 0.2 mM H_2O_2 and apoptotic cell death was measured by flow cytometry. Consistently, shRNA2 was still effective to suppress endogenous Prx-1 in HeLa cells (Fig. 5B, lanes 3). Upon treatment with 0.2 mM H_2O_2, the number of apoptotic cells increased significantly in mock-transfected cells, up to about 26% of the total cells (Fig. 5A, pSUPER retro + H_2O_2), compared with that in H_2O_2-non-treated mock transfected cells (Fig. 5A, pSUPER retro). Interestingly, H_2O_2-induced apoptosis was significantly enhanced in HeLa cells transfected with shRNA2 for Prx-1 compared with that in mock-transfected cells (47 ± 1.5% versus 29 ± 4.7%, respectively) (Fig. 5A). Similar effects could be detected in 293 cells (Fig. 5C and D). These results suggest that Prx-1 potentially functions as an endogenous antagonist against ASK1-mediated apoptosis by H_2O_2.

Based on our findings, we propose a mechanism for the negative regulation of ASK1 activity by Prx-1 (Fig. 5E). Prx-1 may have dual functions in physiological conditions; first, as previously well defined, it might be essential for eliminating H_2O_2 generated by a variety of cellular stresses and proinflammatory cytokines [10–12] and, second, it may have a protective role against ASK1-induced apoptosis mediated by oxidative stresses. When oxidative stresses are transduced into the cells, increased ROS may act on Trx or Prx-1. One of the cellular responses against increased ROS leads to oxidation of Trx and then dissociation of Trx from ASK1. The other cellular response leads to oxidation of Prx-1 to eliminate H_2O_2. If cells do not have other regulatory mechanisms against ASK1 activation, the dissociation of Trx from ASK1 continuously leads to activation of ASK1 and induces ASK1-induced apoptosis. In this regard, oxidized Prx-1 preferentially interacts with ASK1 via the Trx-binding domain of ASK1, and thus inhibits further activation of ASK1. Through this reciprocal interaction of Trx and Prx-1 with ASK via redox-sensitive reactions, cells may effectively respond to oxida-

tive stress-induced cell death, especially ASK1-mediated apoptosis.

It has been reported that ROS-mediated ASK1 activation is involved in a variety of disorders, such as inflammation [13,14], neurodegeneration [15,16], and cardiac hypertrophy and remodeling [17,18]. Therefore, further studies on the mechanisms of regulation of ASK1 activity and the development of ASK1-targeting drugs may contribute to the treatment of various diseases caused by oxidative stress.

Along with these clinical implications of ASK1, Prx-1 may also be targeted in the development of therapeutic drugs to treat diseases. A recent report has shown that Prx-1 expression is elevated in most cancers [19,20], although the physiological means is still undefined. Based on our results, it is tempting to propose that increased Prx-1 in cancer cells may play a critical role in providing resistance against extracellular damages from oxidative stress via inhibition of ASK1 activation and then inhibition of ASK1-induced apoptosis.

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